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Phosphorylation of Histone H3 Is Required for Proper Chromosome Condensation and Segregation

Yi Wei, Lanlan Yu, Josephine Bowen, Martin A. Gorovsky,*† and C. David Allis*†‡
Department of Biology
University of Rochester
Rochester, New York 14627

Summary

Phosphorylation of histone H3 at serine 10 occurs during mitosis in diverse eukaryotes and correlates closely with mitotic and meiotic chromosome condensation. To better understand the function of H3 phosphorylation in vivo, we created strains of *Tetrahymena* in which a mutant H3 gene (S10A) was the only gene encoding the major H3 protein. Although both micronuclei and macronuclei contain H3 in typical nucleosomal structures, defects in nuclear divisions were restricted to mitotically dividing micronuclei; macronuclei, which are amitotic, showed no defects. Strains lacking phosphorylated H3 showed abnormal chromosome segregation, resulting in extensive chromosome loss during mitosis. During meiosis, micronuclei underwent abnormal chromosome condensation and failed to faithfully transmit chromosomes. These results demonstrate that H3 serine 10 phosphorylation is causally linked to chromosome condensation and segregation in vivo and is required for proper chromosome dynamics.

Introduction

The cell division cycle constitutes a series of processes that have evolved to create two genetically identical daughter cells from a mother cell. One of these processes is the conversion of relatively amorphous, extended interphase chromatin into condensed, highly ordered mitotic chromosomes. Proper mitotic chromosome condensation is essential for the correct segregation of sister chromatids into two daughter cells. However, the mechanisms for transition from interphase chromatin into mitotic chromosomes is poorly understood (for review, see Koshland and Strunnikov, 1996).

Recently, SMC (structural maintenance of chromosomes) proteins have been implicated as major players in mitotic chromosome condensation (for review, see Hirano, 1998, 1999; Jessberger et al., 1998; Strunnikov, 1998). In yeast, mutations in SMC proteins were shown to have defects in mitotic chromosome condensation (Saka et al., 1994; Strunnikov et al., 1995). In *Xenopus* egg extracts, immunodepletion of SMC proteins was shown to inhibit chromosome condensation (Hirano and Mitchison, 1994). Two *Xenopus* SMC proteins, XCAP-C and XCAP-E, form a 13S condensin complex with three other proteins (Hirano et al., 1997) that is able to introduce positive supercoils into a relaxed circular DNA in the presence of topoisomerase I and ATP (Kimura and Hirano, 1997). This activity is regulated by Cdc2-dependent phosphorylation during mitosis (Kimura et al., 1998). Thus, the major cell cycle kinase Cdc2 also appears to play a role in integrating mitotic chromosome condensation with the many other events of the cell cycle. However, little is known about the mechanisms by which condensin complexes interact with the other components of chromosomes to effect condensation (for review see Murray, 1998; Hirano, 1999).

In eukaryotic cells, DNA is associated with histone proteins to form chromatin, the basic unit of which is the nucleosome core particle consisting of 146 bp DNA wrapped around an octameric core containing two each of the four conserved core histones H2A, H2B, H3, and H4 (for details, see Luger et al., 1997). A fifth histone, the linker histone H1, interacts with DNA of variable length, links adjacent nucleosome cores, and further compacts the chromatin (for review, see Wolffe et al., 1997; Vignali and Workman, 1998). As a result, H1 has long been assumed to play a role in mitotic chromosome condensation (Bradbury, 1992; Roth and Allis, 1992).

Many recent studies have served to emphasize the importance of posttranslational modifications of histones, notably acetylation, in altering the structure and function of chromatin, particularly during transcription (for review, see Wolffe, 1998). Early experiments demonstrated that major increases in the phosphorylation of histone H1 occurred during mitosis in diverse eukaryotes (Bradbury et al., 1973; Gurley et al., 1978), and this modification is also dependent on the kinase activity of Cdc2 (Langan et al., 1989), leading to the proposal that this modification played an important role in mitotic chromosome condensation (Bradbury, 1992; Roth and Allis, 1992). However, recent experiments have shown that H1 phosphorylation (Guo et al., 1995; Ajiro et al., 1996b), or even H1 itself (Ohsumi et al., 1993; Shen et al., 1995; Patterton et al., 1998), is not necessary for mitotic or meiotic chromosome condensation. Therefore, the significance of mitotic H1 hyperphosphorylation remains unclear.

In contrast to H1 hyperphosphorylation, site-specific phosphorylation of core histone H3 at serine 10 (Ser10) appears to occur exclusively during mitosis in mammalian cells (Gurley et al., 1978; Paulson and Taylor, 1982). H3 phosphorylation, but not H1 hyperphosphorylation, occurs when premature chromatin condensation is induced by treatment of tissue culture cells with the phosphatase inhibitors fostriecin (Guo et al., 1995) or okadaic acid (Ajiro et al., 1996b). Similarly, vanadate-induced dephosphorylation of H3 (but not H1) is temporally correlated with chromatin decondensation in the rescue of a conditional mutant tissue culture cell line that otherwise fails to initiate postmitotic chromatin decondensation at the restrictive temperature (Ajiro et al., 1996a). Also, recent studies employing an antibody specific for H3 phosphorylated at Ser10 have revealed a tight correlation between H3 phosphorylation and mitotic chromatin

*To whom correspondence should be addressed (e-mail: (micro@dialup.rochester.edu [M. A. G.], allis@virginia.edu [C. D. A.]).
†The contributions of the Gorovsky and Allis labs were equal.
‡Present address: Department of Biochemistry and Molecular Genetics, University of Virginia Health Science Center, Charlottesville, Virginia 22908.

condensation in mammalian cells (Hendzel et al., 1997), and this modification is conserved across eukaryotes (Wei and Allis, 1998; Wei et al., 1998). Taken together, the above observations are consistent with the hypothesis that H3 phosphorylation plays an important role in mitotic chromosome condensation.

To date, all studies of H3 phosphorylation and mitosis have been correlative. In higher eukaryotes, the existence of multiple copies of histone (including H3) genes makes it difficult to study H3 phosphorylation directly by genetically manipulating the phosphorylation site in vivo. On the other hand, in yeast, where there are only two copies of the H3 gene, H3 phosphorylation has not been demonstrated, thus precluding the kind of genetic manipulations that have led to greater understanding of the role of acetylation in transcriptional activation and repression (reviewed in Struhl, 1998).

We have developed the ciliated protozoan *Tetrahymena thermophila* as a model for studying the function of H3 phosphorylation in vivo. Like most ciliated protozoa, *Tetrahymena* contains two nuclei: a macronucleus and a micronucleus. During vegetative growth, macronuclei are endoreplicated, transcriptionally active somatic nuclei that divide amitotically. In contrast, micronuclei are diploid, transcriptionally inactive germline nuclei that divide mitotically (Gorovsky et al., 1978). In *Tetrahymena*, H3 phosphorylation is also correlated closely with mitosis in that it occurs only in micronuclei and not in macronuclei of logarithmatically growing vegetative cells (Allis and Gorovsky, 1981). Recently, it was also shown that micronuclear H3 is phosphorylated at a single site within its amino-terminal domain, corresponding to Ser10 in mammalian H3, and that this modification is temporally correlated with chromosome condensation during micronuclear mitosis and meiosis (Wei et al., 1998). Like yeast, *Tetrahymena* has only two genes, *HHT1* and *HHT2*, that encode the identical major H3 protein. A third gene, *HHT3*, encodes an H3.3-like replacement variant that is found only in macronuclei (Bannon et al., 1983; Yu and Gorovsky, 1997).

With the recent development of high-frequency DNA-mediated biolistic transformation (Cassidy-Hanley et al., 1997), it has become possible to perform repeated transformations of the somatic macronucleus in *Tetrahymena* (Yu and Gorovsky, 1997). To investigate the function of H3 phosphorylation in vivo, we used these methods to create strains of *Tetrahymena* that carry a mutant H3 gene (S10A) as the only gene encoding the major H3 protein. We show that phosphorylation of H3 at this conserved site plays an important role in chromosome condensation and segregation during micronuclear mitosis and meiosis. Because these effects are restricted to the mitotic micronucleus that has little or no function in vegetative cells, H3 phosphorylation is not required for vegetative growth in *Tetrahymena*. A similar function for this conserved histone modification in cells whose only nucleus divides mitotically is likely to be essential.

Results

Creation of *Tetrahymena* Strains with Unphosphorylatable Histone H3

In *Tetrahymena*, two genes, *HHT1* and *HHT2*, encode the same major H3 protein that is found in both the mitotic micronucleus and the amitotic macronucleus. A third gene, *HHT3*, encodes an H3.3-like replacement variant that is found only in macronuclei (Bannon et al., 1983; Yu and Gorovsky, 1997). Deletion of either of the major H3 genes does not affect vegetative growth of *Tetrahymena*; deletion of both is lethal (Yu and Gorovsky, 1997). To create a strain of *Tetrahymena* whose major H3 cannot be phosphorylated, we disrupted the *HHT1* gene and replaced the wild-type *HHT2* gene with one in which the codon encoding Ser10 has been changed to encode alanine by in vitro mutagenesis (see Experimental Procedures). The endogenous macronuclear *HHT1* genes were disrupted using a construct in which the *neo2* cassette (Gaertig et al., 1994a) was inserted in place of part of the *HHT1*-coding and 3′-flanking regions (Figure 1A). A 3.2 kb SpeI–ClaI fragment containing the disrupted *HHT1* gene was introduced into *Tetrahymena* cells using biolistic transformation, and complete macronuclear replacements were selected with paromomycin. To create an *HHT2* gene that lacked the Ser10 phosphorylation site, the codon for Ser10 of *HHT2* was changed to encode alanine, which also introduced an AgeI restriction site nearby, and the *neo2* cassette was inserted into the 3′-flanking region as a selectable marker (Figure 1B). A 3.5 kb SpeI–ClaI fragment containing the mutated *HHT2* gene and the selectable marker was then introduced into macronuclei of cells containing the disrupted *HHT1* gene to replace the endogenous *HHT2* gene. By selecting cells with higher concentrations of paromomycin, *HHT1* disruption/*hht2-2S10A* replacement double transformants were obtained. While the above general strategy was used for all S10A transformants, the two different strains used in the studies described here were transformed slightly differently. For strain CU428, transformation was done sequentially, with the *HHT1* gene being completely knocked out before the cells were subsequently transformed with *hht2-2S10A* gene. For strain CU427, the two transforming DNAs were introduced into cells simultaneously.

Gene Disruption and Replacement of *HHT1* and *HHT2*

Because *Tetrahymena* macronuclei are polyploid, only partial replacement of some of the 45 endogenous copies is initially obtained in a typical transformation experiment. However, during vegetative growth, the multiple copies of macronuclear genes are segregated randomly as macronuclei undergo amitotic divisions, a process known as phenotypic assortment (Merriam and Bruns, 1988). Thus, complete replacement of a nonessential gene can eventually be obtained when sufficient selection pressure is applied, even if the transforming gene has a deleterious but nonlethal phenotype. Double transformants can be selected, even if both transforming constructs contain the same selectable marker, as long as the drug resistance exhibited by the double transformant exceeds that of one of the single transformants (Yu and Gorovsky, 1997).

Total cellular DNA isolated from the double transformants (referred to as S10A transformants), as well as DNA from wild-type cells, was digested with HindIII and analyzed on a Southern blot (Figure 1C) by hybridization with probes specific for either *HHT1* (Figure 1A) or *HHT2*

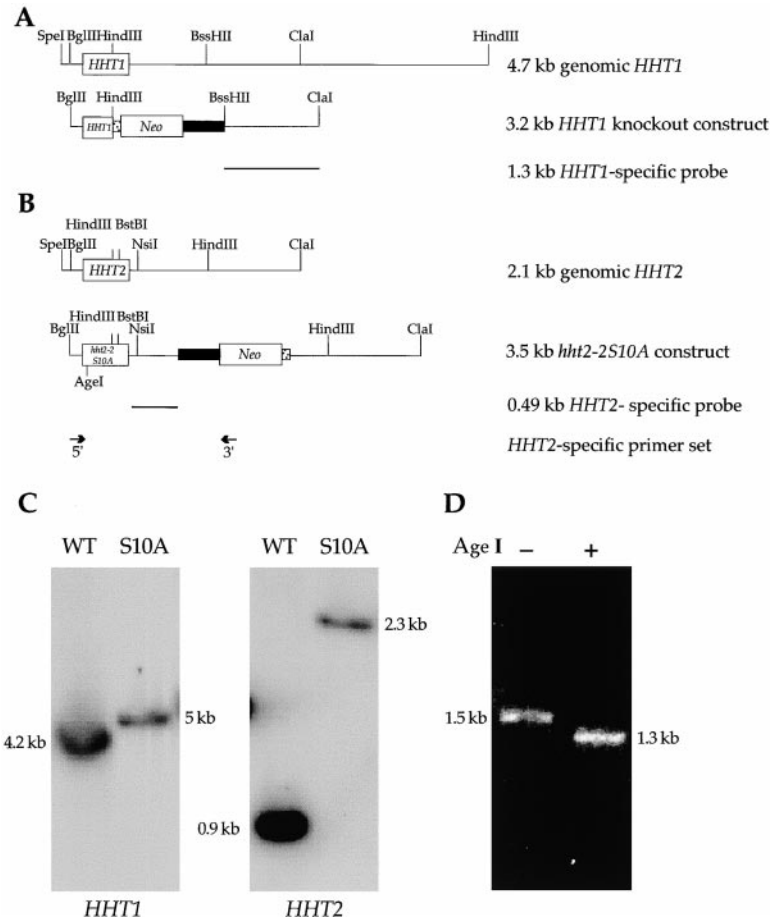

Figure 1. Southern and PCR Analyses of S10A Cells

(A) The macronuclear genomic *HHT1* gene is shown as a 4.7 kb fragment containing the *HHT1* coding region (open box). A 3.2 kb *HHT1* knockout construct is shown. The *neo2* cassette was inserted between the HindIII and BssHII sites. The stippled box is the *HHF1* promoter, followed by the *neo* gene and *Btu2* terminator (solid box). The *neo* gene was transcribed in the same direction as the *HHT1* gene. A 1.3 kb *HHT1*-specific probe is shown at the bottom.

(B) The macronuclear genomic *HHT2* gene is shown as a 2.1 kb fragment. A 3.5 kb *hht2-2S10A* construct is shown. Next to the serine 10 to alanine mutation, an AgeI restriction site was introduced into the *HHT2* coding region (open box). The *neo2* cassette was inserted in the 3′-flanking region of *HHT2* in the opposite orientation as the *HHT2* gene. A 0.49 kb *HHT2*-specific probe is shown, as is a set of *HHT2*-specific primers.

(C) Total genomic DNA from wild-type or S10A cells was digested with HindIII and hybridized with *HHT1*- (left panel) or *HHT2*- (right panel) specific probes.

(D) The HHT2 gene region was PCR amplified from S10A genomic DNA. The PCR product with or without AgeI digestion was run on an agarose gel.

gene (Figure 1B). As shown in Figure 1C, left panel, a 4.2 kb fragment was observed that was derived from the endogenous *HHT1* gene. In the S10A transformants, the 5 kb fragment expected from the disrupted gene was observed, while the 4.2 kb fragment was no longer detectable. This result indicates that all of the endogenous macronuclear *HHT1* genes had been replaced by the disrupted version. Similarly, with the *HHT2* probe, a 0.9 kb fragment derived from the endogenous *HHT2* gene was completely replaced by the expected 2.3 kb band from the transforming *hht2-2S10A* gene, indicating complete replacement of the endogenous gene by the mutated *HHT2* gene (Figure 1C, right panel).

During the time required for phenotypic assortment and drug selection, the newly introduced *hht2-2S10A* genes coexisted with the endogenous *HHT2* genes in the polyploid macronuclei, and it is possible that the two types of *HHT2* genes recombined to introduce the flanking *neo2* cassette into the chromosomes without the S10A mutation. To determine whether this had occurred, the *HHT2* gene region was PCR amplified from genomic DNA of S10A cells and digested with the AgeI restriction endonuclease. As the AgeI restriction site was introduced next to the S10A mutation, the absence of detectable uncleaved DNA from the S10A mutant cells indicates that no recombination had occurred to separate the S10A mutation from the selectable marker and that all of the *HHT2* genes in the S10A cells carried the S10A mutation (Figure 1D, right lane).

Loss of Micronuclear DNA in S10A Cells

The recovery of transformants in which the mutated *HHT2* gene had completely replaced the wild-type *HHT2* gene indicated that phosphorylation of Ser10 of H3 is not essential for vegetative growth of *Tetrahymena*. However, microscopic observation of mutant cells stained with the DNA-specific dye diamidinophenolindole (DAPI) indicated that S10A cells had lost much of their micronuclear DNA, as S10A micronuclei appeared significantly smaller than wild-type micronuclei (Figure 2A). To document this loss, we measured the micronuclear DNA content directly. Both wild-type and S10A cells were lysed with NP-40 and stained with the DNA-specific dye propidium iodide, and the DNA contents of macro- and micronuclei were measured by flow cytometry. Consistent with the DAPI staining result, the S10A micronuclei contained less DNA than the (presumably diploid–tetraploid) wild-type micronuclei (Figure 2B). In contrast, the macronuclear DNA content of S10A cells was greater than that of wild-type cells (Figure 2B). However, the absence of H3 phosphorylation probably did not affect macronuclear division directly. Instead, this change in macronuclear DNA content most likely was a secondary effect of the disruption of micronuclear

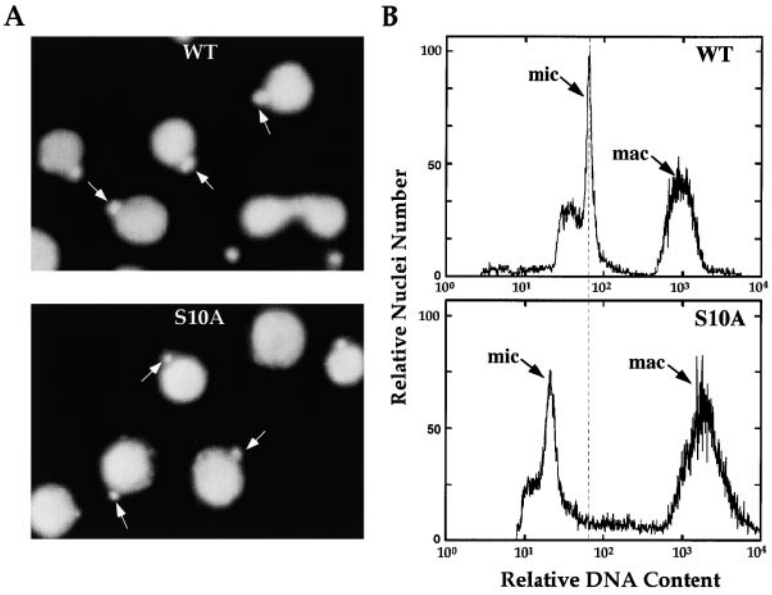

Figure 2. Extensive DNA Loss in S10A Micronuclei

(A) Wild-type (upper panel) or S10A (lower panel) cells were fixed and stained with the DNA-specific dye DAPI. The arrows indicate micronuclei in wild-type and S10A cells. Note that most, if not all, of the micronuclei in the S10A cells are smaller than their counterparts in wild-type cells, a result quantified in (B).

(B) Wild-type (upper panel) or S10A (lower panel) cells were lysed by NP-40 and stained with the DNA-specific dye propidium iodide. Macro- and micronuclear DNA contents were then measured by flow cytometry.

mitosis, as will be discussed below. These results suggest that H3 phosphorylation is essential for the faithful transmission of micronuclear DNA from asexual generation to generation.

Reintroduction of Diploid Micronuclei into S10A Cells

As shown in Figure 2, by the time we examined S10A cells in any detail, their micronuclei had lost considerable DNA and become very small, making it difficult to study the morphology of micronuclear chromosomes and the mechanism leading to micronuclear DNA loss in S10A cells. To overcome this problem, we reintroduced diploid, wild-type micronuclei into S10A cells as a means to "reset the clock" with respect to micronuclear function in our analyses.

In *Tetrahymena*, a special type of abortive mating called genomic exclusion has been shown to occur when wild-type cells are mated with so-called "star" strains that have defective micronuclei (for details, see Figure 3 and Allen, 1967; Doerder and Shabatura, 1980). The star strain can form conjugal pairs with the wild-type strain but fails to produce a gametic nucleus during meiosis. As a result, the normal strain partner contributes a migratory gametic micronucleus to the star cell but receives nothing from its star mate at the fertilization stage of conjugation. The single haploid micronucleus in each conjugant is then endoreplicated, becoming a diploid nucleus homozygous at every locus. The pairs then separate prematurely without performing any of the postzygotic divisions that result in the formation of a new macronucleus and without destroying their old macronucleus. As a result, these cells retain their old macronuclei and their parental phenotypes.

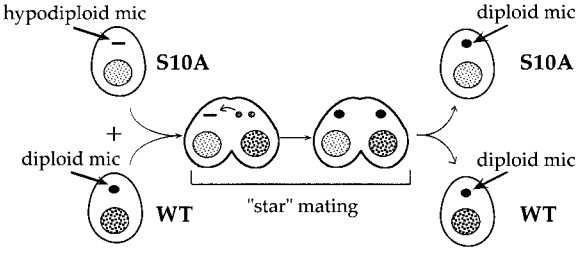

Figure 3. Reintroduction of Diploid, Wild-Type Micronuclei into S10A Cells via Genomic Exclusion

We sought to reintroduce diploid, wild-type micronuclei into S10A cells as a means to "reset the clock" with respect to micronuclear function in our analyses. In *Tetrahymena*, a special type of abortive mating called genomic exclusion has been shown to occur when wild-type cells are mated with so-called "star" strains that have defective, hypodiploid micronuclei. By mating wild-type cells with S10A partners, via this alternative form of genomic exclusion, we have essentially introduced a diploid, wild-type micronucleus into S10A cells (for details, see text).

We reasoned that the S10A transformants might behave in conjugation like the star strains inasmuch as they resembled these strains in having severely hypodiploid micronuclei. This was indeed the case. S10A cells, when mated with wild-type cells, regained a diploid micronucleus. As shown in Figure 4B, shortly after mating, micronuclei in S10A cells appeared similar to wild-type micronuclei when stained with the DNA-specific dye DAPI. They also retained their old macronuclei and the phenotypes associated with it: they were paromomycin resistant (data not shown) and lacked phosphorylation at Ser10 of H3 (see below). All subsequent experiments were done using these S10A cells into which new, diploid micronuclei had been reintroduced. Because these cells still retained the *hht2-2S10A* genes in their macronuclei and their micronuclei quickly came to resemble the micronuclei of the original S10A transformants (see below), we also refer to them as S10A cells.

Absence of H3 Phosphorylation in the Micronuclei of S10A Cells

To determine whether H3 phosphorylation was present in the micronuclei of S10A mutants, we performed Western analysis. Proteins from macro- (Figure 4A, lanes 1 and 3) or micronuclei (Figure 4A, lanes 2 and 4) of either

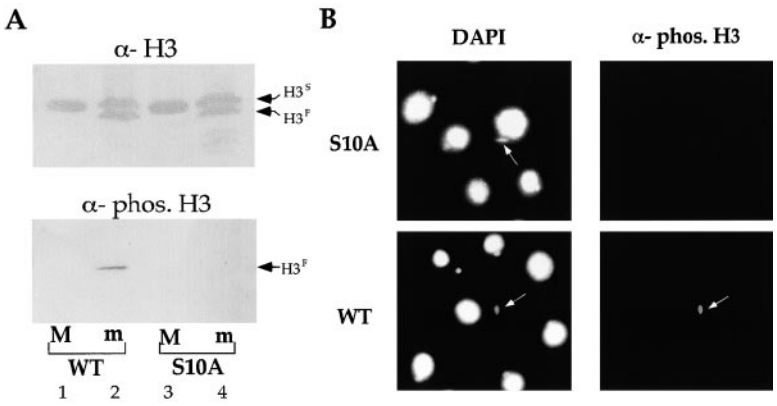

Figure 4. Absence of H3 Phosphorylation in S10A Cells

(A) Proteins from macro- (lanes 1 and 3) or micronuclei (lanes 2 and 4) of wild-type (lanes 1 and 2) and S10A (lanes 3 and 4) cells were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with total H3 (upper panel) or phosphorylated H3 (lower panel) antibodies.

(B) S10A (upper panels) and wild-type (lower panels) cells were fixed and stained with phosphorylated H3 antibody (right panels) or the DNA-specific dye DAPI (left panels). The arrows indicate mitotic micronuclei in wild-type and S10A cells. Note that the football-shaped mitotic micronuclei are always stained in wild-type cells but not in the S10A mutant cells.

wild-type (Figure 4A, lanes 1 and 2) or S10A (Figure 4A, lanes 3 and 4) *Tetrahymena* cells were separated on SDS-PAGE and blotted onto a nitrocellulose membrane. Antibodies that recognize all known forms of major *Tetrahymena* H3 and antibodies specific for phosphorylated H3 were then used to detect total H3 and Ser10-phosphorylated H3, respectively. As described previously, two electrophoretically distinct forms of H3 were observed in micronuclei (Figure 4A, upper panel, lane 2, and Allis et al., 1980). The slower migrating species, $H3^S$, is identical to macronuclear H3, while the faster species, $H3^F$, is derived from $H3^S$ by a proteolytic cleavage that removes the first six amino acids of $H3^S$, generating an isoform that is unique to micronuclei (Allis et al., 1980). In wild-type cells, H3 phosphorylation occurs only in micronuclei and is specific to micronuclear $H3^F$ (Figure 4A, lower panel, lanes 1 and 2, and Allis and Gorovsky, 1981). The S10A mutation in H3 did not affect the proteolytic cleavage, as two forms of H3, $H3^S$ and $H3^F$, were still present in S10A micronuclei (Figure 4A, upper panel, lane 4). However, no phosphorylated H3 was detected in either macro- or micronuclei of S10A cells (Figure 4A, lower panel, lanes 3 and 4).

The absence of H3 phosphorylation was further demonstrated by in situ staining using the phosphorylated H3 antibody. As shown previously (Wei et al., 1998), in wild-type cells, mitotic micronuclei, which have a characteristic "football" shape, were invariably stained by the phosphorylated H3 antibody (Figure 4B, lower panels). In contrast, micronuclei with a similar mitotic football shape in S10A cells failed to be stained by the antibody (Figure 4B, upper panels).

Abnormal Micronuclear Mitosis in S10A Cells

As shown in Figure 5A, S10A cells grew slightly slower than wild-type cells (doubling time, 3.1 hr versus 2.5 hr). However, S10A cells did not show prolonged arrest in any of the cell cycle stages. As H3 phosphorylation had been shown to occur only during micronuclear mitosis in vegetatively growing cells (Wei et al., 1998), we examined further the fidelity of mitosis in micronuclei of S10A cells.

The *Tetrahymena* cell cycle is somewhat unusual in that the micronucleus and the macronucleus divide by different mechanisms (i.e., mitotically versus amitotically) and at different times in the cell cycle. In wild-type cells, micronuclei enter mitosis and display the characteristic football shape before signs of macronuclear division are obvious (Figure 5B, panel a). Micronuclear mitosis is completed before macronuclei begin to divide, and by the time macronuclei enter division, the two daughter micronuclei have clearly separated

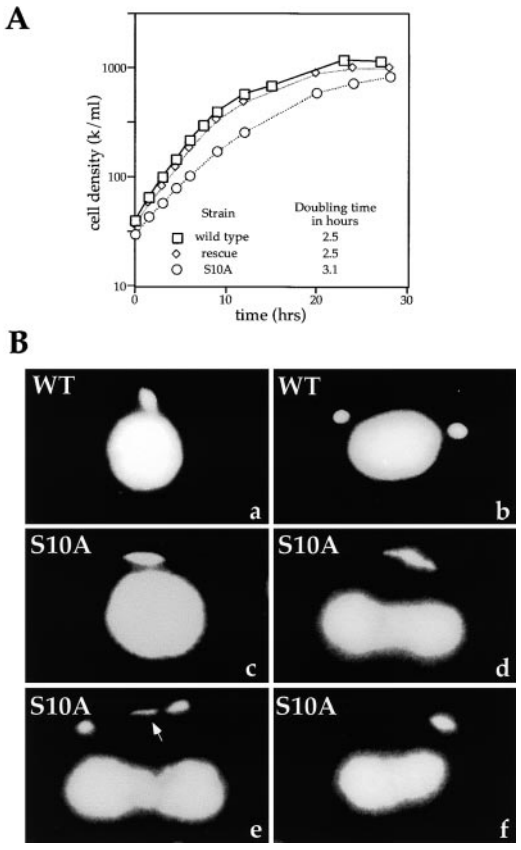

Figure 5. Abnormal Micronuclear Mitosis in S10A Cells

(A) Cells were grown in 1×SPP medium at 30°C. Cell densities were measured up to 30 hr and plotted on a log scale. Doubling time in hours is listed.

(B) Logarithmatically growing wild-type and S10A cells were fixed and stained with the DNA-specific dye DAPI. Normal micronuclear division and macronuclear division in wild-type cells is shown in (a) and (b). Examples of abnormal micronuclear division are shown in (c–f). The arrow indicates lagging chromosomes between the two separating daughter nuclei.

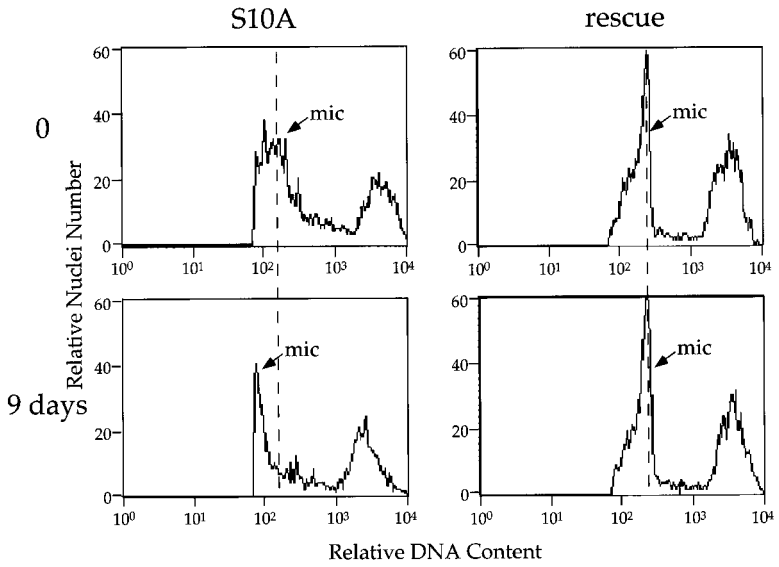

Figure 6. Progressive Loss of S10A Micronuclear DNA during Vegetative Growth

S10A (left panels) and rescue (right panels) cells were kept in logarithmic growth by transferring to fresh 1×SPP medium every 24 hr. At time point 0 (upper panels) and after 9 days of growth (lower panels), cell samples were removed, lysed, and stained with the DNA-specific dye propidium iodide. Macronuclear and micronuclear DNA contents were then determined by flow cytometry.

into the opposite ends of the dividing cell (Figure 5B, panel b). Compared with wild-type cells, mitotic micronuclei in S10A cells appeared to be more elongated (Figure 5B, panel c), suggesting that they had difficulty getting through mitosis. In fact, some S10A micronuclei were so delayed in mitosis that they were still undergoing mitosis—as shown by the football-shaped micronuclei—when macronuclear division had initiated (Figure 5B, panel d). This delay in mitosis could lead to complete failure of chromosome segregation, as suggested by observations of a dividing micronucleus entirely within one-half of a dividing cell (Figure 5B, panel f). Alternatively, partial failure of segregation is indicated in Figure 5B, panel e, where most micronuclear chromatin was well separated but some chromosomes were lagging between the two separating daughter nuclei. These phenotypes are reminiscent of the "cut" phenotype in the fission yeast *S. pombe* (for review, see Yanagida, 1998), suggesting that absence of H3 phosphorylation causes defects in mitotic chromosome segregation and possibly in mitotic chromosome condensation.

Progressive Loss of S10A Micronuclear DNA during Vegetative Growth

The above results suggest that the loss of micronuclear DNA, as observed in Figure 2, was caused by abnormal mitotic chromosome segregation, in which case it should become progressively worse with successive cell divisions. To test this, S10A cells were kept in continuous logarithmic growth by transferring cells into fresh growth medium every 24 hr. At both time point 0 and after 9 days of continuous growth, samples were removed, and the DNA content of macro- and micronuclei was measured by flow cytometry as described above. As shown in the left panels of Figure 6, in S10A cells the micronuclear DNA content decreased progressively in continuous growth. This result argues strongly that the loss of micronuclear DNA is dependent on cell divisions.

In contrast to micronuclei, where failure to phosphorylate H3 causes chromosome segregation errors that accumulate after successive mitotic divisions, resulting in extensive chromosome loss, in macronuclei, which divide amitotically, chromosome missegregation and subsequent chromosome loss did not occur. In fact, macronuclear DNA content in S10A cells was greater than in wild-type cells (Figure 2B), probably as a result of perturbations in the cell cycle resulting from micronuclear division delays and the decoupling of micro- and macronuclear divisions, as shown in Figure 5B. As only macronuclei are transcriptionally active during vegetative growth and micronuclei are dispensable, S10A cells missing partial or complete micronuclear chromosomes are still viable. This is not the case for most cells whose only nucleus divides mitotically; here chromosome missegregation is expected to be lethal.

Abnormal Micronuclear Meiosis in S10A Cells

Micronuclear chromosomes do not condense fully and display themselves distinctively during mitosis, making it difficult to assess mitotic chromosome condensation in *Tetrahymena*. Fortunately, micronuclear chromatin does form more typical chromosomes during meiosis, enabling more detailed analysis. Micronuclear meiosis occurs during conjugation, shortly after cell pairing. At the end of meiotic prophase, five chromosome bivalents, formed by the pairing of maternal and paternal chromosomes, enter diakinesis and become highly condensed. During this period, H3 phosphorylation has been shown to occur in a fashion that closely coincides with meiotic chromosome condensation (Wei et al., 1998).

When two S10A cells of different mating type were mated, condensed chromosome bivalents formed (Figure 7B), but some showed clear defects in meiotic chromosome condensation. Figure 7B shows a chromosome bivalent whose middle portion was fully condensed while its two ends (arrows) still remained in an extended form. Other cells showed what appear to be anaphase bridges connecting the two products of the first meiotic division (Figure 7D, arrowhead). These abnormalities have not been observed in wild-type matings (Figures 7A and 7C). These results indicate that while the S10A

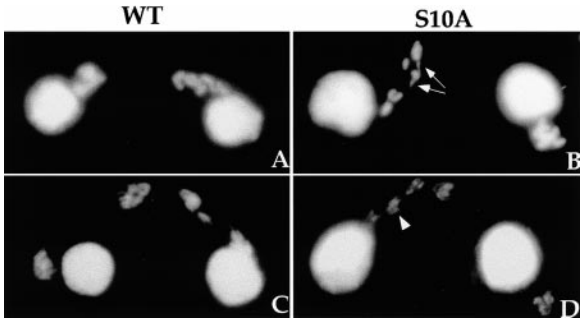

Figure 7. Abnormal Meiotic Chromosome Condensation and Segregation without H3 Phosphorylation

Wild-type (A and C) and S10A (B and D) cells of different mating types were mated, fixed, and stained with the DNA-specific dye DAPI. The arrows indicate portions of an S10A chromosome bivalent that remained in extended form. The arrowhead denotes S10A chromosomes that lagged in meiosis, forming an anaphase bridge.

mutation in H3 does not disrupt chromosome condensation completely, it does cause defects in meiotic chromosome condensation and segregation.

Rescue of S10A Phenotypes by the Wild-Type *HHT2* Gene

As the generation of S10A transformants required a prolonged period of drug selection, it is possible that extra mutations occurred in the genome and the phenotypes we observed were the result of these mutations instead of changes in H3 phosphorylation state. To exclude this possibility, we tried to rescue the S10A phenotypes by reintroducing the wild-type *HHT2* gene into S10A cells. As shown in Figure 5A, the rescue strain with diploid micronuclei grew as fast as the original wild-type cells. Also shown in Figure 6, right panels, the rescue cells retained their micronuclear DNA content after continuous vegetative growth. In addition, the behavior of rescue micronuclei in mitosis and meiosis was similar to that of wild-type micronuclei (data not shown). Taken all together, the above results suggest that the abnormal mitosis and meiosis we observed in S10A cells was caused by the absence of H3 phosphorylation and not by extra mutations in the genome.

Discussion

H3 phosphorylation has long been correlated with mitosis both temporally and spatially. Recent studies employing a novel Ser10-phosphorylated H3 antibody have shown, for example, that H3 phosphorylation is closely associated with mitotic and meiotic chromosome condensation (Hendzel et al., 1997; Wei et al., 1998). Furthermore, in cases where linker H1 hyperphosphorylation is decoupled from mitotic chromosome condensation, H3 phosphorylation still occurs (Guo et al., 1995; Ajiro et al., 1996a, 1996b). Nevertheless, all of these above observations underscore a correlative but not necessarily causal relationship between H3 phosphorylation and mitosis, and similar correlations between H1 phosphorylation and mitosis have proved to be misleading.

In the studies described here, we utilized the specificity of in vitro mutagenesis coupled with complete gene replacement to restrict our analysis to the single, conserved site of H3 phosphorylation. We demonstrate that H3 phosphorylation at Ser10 is essential for proper chromosome dynamics during mitosis in vivo. Absence of H3 phosphorylation in *Tetrahymena* causes improper chromosome segregation, and this improper segregation is probably preceded by defects in chromosome condensation, defects that are most clearly evident during meiotic chromosome condensation. It is worth noting that the chromosome loss that accompanies the absence of H3 phosphorylation would probably have made it difficult to demonstrate the function of this modification in most biological systems where the resulting aneuploidy would almost certainly have been lethal. However, in *Tetrahymena* this loss is restricted to the mitotic micronucleus that is transcriptionally silent in vegetative cells and therefore dispensable.

Unlike a recently described *Tetrahymena* telomere mutant, whose defect in chromosome segregation leads to arrest in anaphase (Kirk et al., 1997), chromosome missegregation caused by a failure to phosphorylate H3 occurs without complete disruption of either nuclear or cytoplasmic divisions. Typically, S10A cells exhibit difficulty in getting through anaphase, as shown by elongated mitotic nuclei and a pronounced delay of micronuclear mitosis relative to macronuclear division. In the most extreme cases, this delay in mitosis causes a complete failure of chromosome segregation, as dividing micronuclei have been observed entirely within one-half of a dividing cell. More often, sets of sister chromosomes are well separated from each other, but some chromosomes were observed lagging between the two daughter nuclei, resulting in a partial failure of segregation.

In this respect, our results differ from conclusions recently reached by in vitro studies where abolishment of H3 phosphorylation, by inhibiting the H3 kinase(s), prevented the initiation of chromosome condensation and entry into mitosis (Hooser et al., 1998). However, in these studies complete failure to induce chromosome condensation may not be due solely to the absence of H3 phosphorylation, as the responsible kinase is likely to have other targets, and these may be essential for chromosome condensation (e.g., condensin-mediated activity appears to be regulated by phosphorylation; Kimura et al., 1998).

The kinase responsible for mitotic H3 phosphorylation is not known. However, it is unlikely that a cyclin-dependent kinase, or any other proline-directed kinase, is directly responsible for mitotic H3 phosphorylation at Ser10. Ser10 in H3 lacks a nearby proline and is not phosphorylated by Cdc2 kinase in vitro (Sweet and Allis, 1993). Also, this kinase is not present in mitotic micronuclei in *Tetrahymena* (Sweet et al., 1997). Ser10 in H3 is surrounded closely by a high density of positively charged residues, a fact consistent with the observation that phosphorylation of Ser10 in H3 is catalyzed efficiently by cyclic AMP–dependent kinase (or PKA) in vitro (Paulson and Taylor, 1982; Hendzel et al., 1997). Recently, it has been suggested that follicle-stimulating hormone (FSH) induced H3 phosphorylation on Ser10, although a separate event from mitotic H3 phosphorylation, is mediated by PKA (DeManno et al., 1999). Moreover, we have determined that Rsk2, a member of the

pp90^{rsk} family of kinases implicated in growth control, is required for mitogen-stimulated H3 phosphorylation at Ser10 (Mizzen et al., 1998; Sassone-Corsi et al., submitted). Interestingly, the pp90^{rsk} family of kinases, like PKA, also prefers basic-directed sites, including Ser10 in H3. The relationship, if any, between mitotic and transcription-related H3 phosphorylation and the responsible kinases remains obscure.

What role, then, does phosphorylation of H3 at Ser10 play in mitosis and meiosis? Defects in chromosome segregation may be a secondary effect due to subtle disturbances in chromosome condensation, as has been shown previously (Saka et al., 1994; Strunnikov et al., 1995). Alternatively, H3 phosphorylation may play a more direct role in chromosome segregation. Previously it has been shown, for example, that a yeast protein, MCD1/Scc1, plays a role in both chromosome condensation and segregation (Guacci et al., 1997; reviewed in Strunnikov, 1998). Recent data in yeast indicate that a variant H3 family member, Cse4p, is also involved in chromosome segregation, presumably by organizing a specialized nucleosome at the centromere (Meluh et al., 1998). Whether Csep4 or its mammalian counterpart, CENP-A (Sullivan et al., 1994), is phosphorylated in vivo at a site(s) resembling Ser10 in the major H3 is not known. Recent evidence suggests that phosphorylation of the major H3 at Ser10 initiates in pericentromeric heterochromatin in many mammalian cells (Hendzel et al., 1997). It is not known whether this H3 phosphorylation event is in any way linked to Cse4p/CENP-A protein function at the centromere.

We propose two models, not mutually exclusive, that could link H3 phosphorylation to SMC and other proteins known to be involved in mitotic chromosome condensation and segregation. Like models proposed for H1 phosphorylation (Roth and Allis, 1992), phosphorylation of H3 may cause a local and transient decondensation of chromatin by introducing a negative charge into the positively charged H3 amino terminus, thereby reducing the interaction between H3 amino termini and DNA. Here, it is envisioned that local decondensation facilitates the binding of condensation factors such as SMC proteins to chromatin, thereby promoting mitotic chromosome condensation.

This model is supported by the fact that absence of H3 phosphorylation does not completely block chromosome condensation, causing only minor defects in some chromosomes. Absence of H3 phosphorylation could make it more difficult for condensation factors to access a chromatin environment without totally excluding their access. Such a model could also explain why H3 phosphorylation also accompanies immediate-early gene transcription in response to mitogen stimulation (Mahadevan et al., 1991; Barratt et al., 1994), PKA-dependent gene transcription in FSH-induced granulosa cell differentiation (DeManno et al., 1999), and decondensation of chromatin in sea urchin sperm pronuclei during fertilization (Vacquier et al., 1989).

Alternatively, phosphorylated H3 could act as a direct ligand or "receptor" for the binding of condensation factors. In this model, condensation factor binding to chromatin would be dependent upon Ser10 phosphorylation within the amino terminus of H3, a process initiated by the yet unidentified mitotic H3 kinase. In this case, the failure to phosphorylate at Ser10 in H3 would be expected to have a more major effect on the initiation of mitotic chromosome condensation H3 unless this phosphorylation event overlaps functionally with phosphorylation or some other secondary modification that reduces the positive charge of one of the other core histones. We look forward to biochemical and genetic tests of these and other models.

Experimental Procedures

Strains, Culture, and Conjugation

T. thermophila strains CU428: *mpr1-1/mpr1-1* (*MPR1*, mp-s, VII) and CU427: *chx-1/chx-1* (*CHX1*, cy-s, VI) were kindly provided by P. J. Bruns (Cornell University). Strains S10A(CU428): * (*MPR1*; *hht1-1::neo2*; *hht2-2S10A*; mp-s, pm-r, VII), S10A(CU427): * (*CHX1*; *hht1-1::neo2*; *hht2-2S10A*; cy-s, pm-r, VI), and rescue(CU428): *chx-1/chx-1*; *HHT1/HHT1*; *HHT2/HHT2*; *RPL29/RPL29* (*MPR1*; *CHX1*; *hht1-1::neo2*; *HHT2*; *RPL29/rpl29-1*; mp-s, cy-r, pm-r, VII) were generated as described below. For studies in vegetative growth, CU428, S10A(CU428), and rescue(CU428) were used. Cells were grown in SPP medium containing 1% Proteose Peptone (1×SPP) (Gorovsky et al., 1975). To analyze growth rates, cells were inoculated in 50 ml of SPP medium and grown at 30°C with vigorous shaking. Cells were counted in a Coulter counter (Coulter Electronics, Inc.) at different time intervals. Growth data were plotted using Cricket Graph III (Computer Associates). Doubling time was calculated using the linear part of the logarithmic growth curve. For conjugation, two strains of different mating types [CU428 with CU427 or S10A(CU428) with S10A(CU427)] were washed, starved (16–24 hr, 30°C), and mated in 10 mM Tris HCl (pH 7.5) as described by Allis and Dennison (1982).

Plasmid Constructions

Plasmid p23B4A2, a pBluescript KS (+) derivative, contains a copy of the *Tetrahymena* genomic *HHT1* gene. To disrupt the *HHT1* gene, a HindIII–SmaI fragment containing the *neo2* gene cassette (Gaertig et al., 1994a) was inserted into p23B4A2 to replace the HindIII–BssHII fragment. This insertion deletes the *HHT1* coding sequence after the HindIII site. The final *hht1-1::neo2* construct was released by digestion with SpeI and ClaI. Plasmid p2C3A, a pBluescript KS (+) derivative, contains a copy of *Tetrahymena* genomic *HHT2* gene. To mutate the *HHT2* gene, a primer, 5′-ACTGCTAGAAAGGCTACCG GTGCTGCTAAGGCC-3′, was used in site-directed mutagenesis (Kunkel, 1985) to change the codon TCC, which encodes Ser10 of *HHT2*, to codon GCT, which encodes alanine. This primer also introduced an AgeI restriction site adjacent to the codon GCT. A *neo2* gene cassette (Gaertig et al., 1994a) was inserted into the 3′-flanking region of the *HHT2* gene. The resulting construct, *hht2-2S10A*, in which the *neo* gene was transcribed in the opposite direction as the *HHT2* gene, was released by digestion with SpeI and ClaI. Plasmid pHHF2HHT2, also a pBluescript KS (+) derivative, contains *Tetrahymena* genomic *HHF2* and *HHT2* genes. The *HHT2* gene was released by digestion with AccI. Plasmid pL29A3 containing a cycloheximide resistance gene, *rpl29-1*, was obtained from P. J. Bruns (Cornell University). The *rpl29-1* gene was released by digestion with HindIII.

Gene Replacement by Biolistic Transformation

Starved vegetative cells were transformed by using the DuPont Biolistic PDS-1000/He Particle delivery system (Bio-Rad), as described previously (Cassidy-Hanley et al., 1997). To generate S10A(CU428), CU428 cells were first transformed with the *hht1-1::neo2* construct and selected with paromomycin, starting from 120 μg/ml to a final concentration of 900 μg/ml, above which the cells failed to grow. The *HHT1* knockout cells were then transformed with the *hht2-2S10A* construct and selected with paromomycin from 1.6 mg/ml to a final 100 mg/ml. S10A(CU427) cells were generated by simultaneous transformation of CU427 cells with both *hht1-1::neo2* and *hht2-2S10A* constructs. The double transformants were selected by paromomycin, starting from 120 μg/ml. To generate rescue(CU428) cells, S10A(CU428) cells were transformed with both wild-type *HHT2* and *rpl29-1* genes (which confers resistance to

cycloheximide), and the transformants were selected with 15 μg/ml cycloheximide. As the wild-type cells had a growth advantage compared to S10A cells, cells containing the wild-type *HHT2* gene cotransformed into S10A(CU428) cells were selected during vegetative growth over those containing *hht2-2S10A* genes. After an extended period of vegetative growth, single cells were selected and cell lines in which *HHT2* had completely replaced *hht2-2S10A* were screened by Southern blotting. To reintroduce diploid micronuclei, S10A(CU428), S10A(CU427), and rescue(CU428) cells with hypodiploid micronuclei were mated with CU427, CU428, and CU427 cells, respectively, pairs were isolated, and paromomycin-resistant partners of the pair were selected (for details, see Results). Cells that had undergone an abortive mating process, genomic exclusion, were identified by observing the sizes of their micronuclei after DAPI staining and the demonstration that they contained diploid amounts of DNA by flow cytometric analysis (see below).

Southern Blot Analysis

Total genomic DNA was isolated as described previously (Gaertig et al., 1994b). Genomic DNA (10 μg) was digested with HindIII and analyzed as described (Gaertig et al., 1993). A 1.3 kb BssHII–ClaI fragment containing the 3′-flanking region of the *HHT1* gene was used as an *HHT1*-specific probe. A 492 bp SpeI–SspI fragment containing the *HHT2* 3′-flanking sequence was used as an *HHT2*-specific probe. Both probes were labeled with [α-^{32}P]dATP by random priming (Ausubel et al., 1988).

PCR Analysis

The *HHT2* gene region of S10A *Tetrahymena* cells was amplified from total genomic DNA by PCR using the following two primers: 5′-CCCGATTCGCAGCGCATCG-3′ (neo specific) and 5′-TCCAGGA TTATGCCAAAC-3′ (HHT2 specific). The PCR product was then digested with AgeI and run on an agarose gel.

Nuclei Preparation

Macro- and micronuclei were isolated from *Tetrahymena* as described by Gorovsky et al. (1975) except that the nucleus isolation buffer contained 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate, and 200 μM chloromeruriphenylsulfonic acid, but not spermidine. Nuclei were boiled in SDS loading buffer for 3 min before loading.

Electrophoresis and Immunoblotting

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and immunoblotting analyses were performed as described previously (Lin et al., 1989). Phosphorylated H3 (Ser10) antibody was used as described previously (Wei et al., 1998) and is available from Upstate Biotechnology Inc., Lake Placid, NY (catalog number 06-570).

Indirect Immunofluorescence Analysis

Growing or conjugating cells were fixed and processed for indirect immunofluorescence as described previously (Wenkert and Allis, 1984). Cells were stained with the DNA-specific dye DAPI at 0.3 μg/ml in Tris-buffered saline (TBS).

Flow Cytometry Analysis

Logarithmatically growing *Tetrahymena* cells were lysed in 0.25 M sucrose, 10 mM $MgCl_2$, and 0.5% NP-40 at a concentration of 1.5×10^6 cells/ml. The DNA-specific dye propidium iodide was then added to 50 μg/ml, and nuclei were stained for 1 hr before flow cytometry analysis using either a Coulter Profiler or a Becton Dickinson Calibur flow cytometer.

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